High performance liquid chromatography profiling of health-promoting phytochemicals and evaluation of antioxidant, anti-lipoxygenase, iron chelating and anti-glucosidase activities of wetland macrophytes

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ABSTRACT

Background: The phytochemistry and bioactivity of wetland macrophytes are underexplored. Plants are known as the natural sources of phytochemical beneficial to health. **Objective:** The objective of this study is to analyze the phytochemical profiles and bioactivities of 10 extracts prepared from different plant parts of wetland macrophytes *Hanguana malayana*, *Ludwigia adscendens* and *Monochoria hastata.* **Materials and Methods:** High performance liquid chromatography (HPLC) was used to analyze the phytochemical profile of the extracts. Antioxidant assay such as 2,2-diphenyl-1-picrylhydrazyl, nitric oxide (NO) radical scavenging activity and ferric reducing antioxidant power were performed. Bioactivity assays carried out were anti-lipoxygenase, anti-glucosidase, and iron chelating. **Results:** Leaf extract of *L. adscendens* had the highest 2,2-diphenyl-1-picrylhydrazyl (half of maximal effective concentration [EC_{50}] = 0.97 mg/mL) and NO (EC_{50} = 0.31 mg/mL) scavenging activities. The extract also exhibited the highest iron chelating (EC_{50} = 3.24 mg/mL) and anti-glucosidase (EC_{50} = 27.5 μg/mL) activities. The anti-glucosidase activity of *L. adscendens* leaf extract was comparable or superior to those of acarbose, myricetin and quercetin. Correlation between iron chelating and radical scavenging activities among the extracts implies the presence of dual-function phytoconstituents with concurrent iron chelating and radical scavenging activities. HPLC analysis revealed the presence of *p*-coumaric acid (*p*-CA), gallic acid (GA) and myricetin in all or most extracts. *M. hastata* fruit and leaf extracts had the highest *p*-hydroxybenzoic acid content. Antioxidant and anti-glucosidase activities of the extracts were correlated with *p*-CA, GA, and myricetin contents. **Conclusion:** Our study demonstrated that wetland macrophytes *H. malayana*, *L. adscendens* and *M. hastata* are potential sources of health-promoting phytochemicals with potent therapeutically-relevant bioactivities.

Key words: Anti-glucosidase, anti-lipoxygenase, antioxidant, high performance liquid chromatography, iron chelating, phytochemical

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INTRODUCTION

Macrophytes are plants that grow either completely or partially submerged or floating on water. Most macrophytes grow naturally in the wild; but, some are cultivated and consumed by humans as plant food. Worldwide, various wetland macrophytes are used traditionally to treat human diseases.[1-4] Wetland macrophytes are a potential natural resource for the discovery of therapeutically-relevant natural products. To date, the health-promoting phytochemicals and bioactivities of many macrophytes are underexplored.

Phenolic constituents of plants are a prominent source of health-promoting phytochemicals. Plant phenolic compounds are structurally diverse and can be divided into different classes, including hydroxybenzoic acids (HBAs), hydroxycinnamic acids, and flavonoids.[5,6] Plant phenolics are known to have numerous health-promoting or therapeutically-relevant effects. Gallic acid, a HBA, can inhibit the formation of reactive oxygen species (ROS) in RAW264.7 mouse macrophages.[7] *p*-coumaric acid (*p*-CA), a

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hydroxycinnamic acid, is considered a potential antidiabetic agent for the prevention or improvement of insulin resistance and type II diabetes.[8] Ferulic acid, another hydroxycinnamic acid, was reported to have anti-inflammatory activity in animal models.[9] Iron-mediated ROS generation and oxidative damage in body cells are associated with health hazards such as cancer.[10] Phytochemicals with the metal-chelating ability are potentially useful for the treatment and/or prevention of iron-mediated pathological conditions.[5] One example of such phytochemicals is myricetin, a flavonoid compound.[11] Myricetin is also known to have anti-lipoxygenase (LOX) activity.[12] LOX is an enzyme that triggers inflammatory mediators such as cyclooxygenase (COX) to initiate the process of inflammation in the body.[13]

Hanguana malayana, *Ludwigia adscendens,* and *Monochoria hastata* are three wetland macrophytes which grow in the tropics. Traditionally, *H. malayana* is used externally to treat fever;[14] macerated leaf of *L. adscendens* is used to treat diarrhea and relieve gastrointestinal disorder; *M. hastata* juice is used to treat boils and drunk as tonic.[4] At present, there is little information in the literature on the therapeutically-relevant bioactivities and phytochemical profiles of the three macrophytes. To the best of our knowledge, while phytochemistry of *L. adscendens* have been reported,[1,15] not much is known about the phytochemical profiles of *H. malayana* and *M. hastata*.

To fill in the current gaps of knowledge on wetland macrophytes, specifically *H. malayana*, *L. adscendens* and *M. hastata*, we have carried out this investigation with the following objectives. First, to determine the phytochemical profiles of *H. malayana*, *L. adscendens*, and *M. hastata*, with special attention on HBAs, hydroxycinnamic acids, and flavonoids. Second, to evaluate the antioxidant, anti-LOX, iron chelating and anti-glucosidase of the three macrophytes. Third, to assess if there were any correlations between the phytochemical contents of the macrophytes and their bioactivities.

MATERIALS AND METHODS

Collection of plant samples and species identification

Specimens of three wetland macrophytes *H. malayana* (family Hanguanaceae), *L. adscendens* (family Onagraceae), and *M. hastata* (family Pontederiaceae) were collected from wetland in the vicinity of the university campus. The plant specimens were authenticated by H.-C. Ong. Voucher herbarium specimens were deposited at the university's herbarium, for future reference.

Preparation of aqueous extracts

Whole plants of *H. malayana, L. adscendens,* and *M. hastata* were washed thoroughly and separated into different plant parts. Table 1 lists the plant parts taken from each specimen for the preparation of 10 aqueous extracts that were analyzed in this investigation. The plant samples were oven-dried at 45°C for 48 h, and then pulverized to powder using a Waring blender. Aqueous extracts were prepared by suspending the plant powder in deionized water at a 1:20 (dry weight: volume) ratio, followed by incubation in a water bath at 95°C with constant agitation at 120 rpm for 2 h. The extracts were vacuum-filtered through cheesecloth. The filtrates were then centrifuged at 9000 rpm and 4°C for 10 min. The supernatant obtained, taken as 50 mg dry matter (DM)/mL in concentration, was aliquoted (500 μL each) and stored at -20°C until used.

High performance liquid chromatography analysis

High performance liquid chromatography (HPLC) analysis was performed using Shimadzu LC-20D dual binary pumps, Shimadzu CTO-10AS column oven, and Shimadzu Prominence SPD-20A UV/Vis detector. The analysis was performed using a C-18 reversed phase column (Phenomenex, Gemini 5 μ, 150 mm length × 4.6 mm internal diameter). The composition of solvents and the gradient elution profile used in this analysis were as described by[16,17] with slight modifications The mobile phase consisted of acetic acid-acidified deionized water (pH 2.8) as solvent A and acetonitrile as solvent B at a flow rate of 0.8 mL/min. Gradient elution was executed as follows: 0-5 min, 5-9% solvent B; 5-15 min, 9% solvent B; 15-22 min, 9-11% solvent B; 22-38 min, 11-18% solvent B; 38-43 min, 18-23% solvent B; 43-44 min 23-90% solvent B; 44-45 min, 90-80%, solvent B; 45-55 min, 80% solvent B; 55-60 min, and 80-5% solvent B. The column was equilibrated with 5% solvent B for 20 min after each injection of samples. The column temperature was set to 38°C and the injection volume was 20 μL. The wavelengths were set to 280 nm for the detection of HBAs, 320 nm for hydroxycinnamic acids, and 370 nm for flavonoids.[17] Phenolic compound identification and quantification were performed by comparing respective retention times and peak areas with pure standard compounds utilizing the method of external standards to construct calibration curve. The concentrations of standards used for calibration curve ranged from 0.01 mM to 3 mM. Table 2 shows the list of phenolic constituents analyzed with HPLC and their retention times.

Table 1: Plant parts used for the preparation of extracts

Macrophytes species	Plant parts used for extract preparation
H. malayana	Leaf, rhizome
L. adscendens	Leaf, stem, root
M. hastata	Leaf, stem, rhizome, root, fruit

H. malayana: Hanguana malayana; L. adscendens: Ludwigia adscendens; M. hastata: Monochoria hastata

Antioxidant assays

Antioxidant activities of the plant extracts were assessed based on three parameters: 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, nitric oxide (NO) scavenging activity, and ferric reducing antioxidant power (FRAP). A previously described DPPH scavenging assay[18] was modified into a microplate format. Briefly, 10 μL of extract was added to 300 μL of 0.004% (w/v) methanolic DPPH. The mixture was incubated in darkness for 30 min at room temperature and the absorbance was measured against a reaction blank at 517 nm. DPPH scavenging activity was calculated using the formula below:

$$\text{DPPH scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control})] \times 100$$

$A_{control}$ is the absorbance of the reaction mixture where the plant extract was omitted. A_{sample} is the absorbance of the reaction mixture where the plant extract was added. Extracts were analyzed in the concentration range of 0-50 mg/mL. Half of maximal effective concentration (EC_{50}) value, defined as the extract concentration required to achieve 50% of DPPH scavenging activity, was determined by using linear regression analysis. Ascorbic acid (Asc) and butylated hydroxytoluene (BHT) were used as positive controls in this assay.

Nitric oxide scavenging activity of plant extracts was determined by a microplate assay modified from.[19] First, 90 μL of extract was pipetted into each well, to which 30 μL of 5 mM sodium nitroprusside in phosphate buffer saline (pH 7.4) was added. The mixture was incubated under fluorescent light at room temperature for 150 min. Then, 90 μL of freshly prepared Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 5% phosphoric acid) was added into the mixture. After 10 min incubation in the dark, the absorbance of the mixture was determined at 560 nm. NO scavenging activity was calculated using the formula below:

$$\text{NO scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control})] \times 100$$

$A_{control}$ is the absorbance of the reaction mixture where the plant extract was omitted. A_{sample} is the absorbance of the reaction mixture where the plant extract was added. Extracts were analyzed in the concentration range of 0-50 mg/mL. EC_{50} value, defined as the extract concentration required to achieve 50% of NO scavenging activity, was determined by using linear regression analysis. Asc was used as positive control in this assay.

Ferric reducing antioxidant power assay measures the ability of a reducing agent to convert ferric tripyridyltriazine (Fe[III]-TPTZ) to ferrous TPTZ (Fe[II]-TPTZ) at low pH. FRAP values of the plant extracts were determined by using a microplate assay modified from.[20] FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-TPTZ-s- in 40 mM HCl and 20 mM $FeCl_3.6\ H_2O$ in a ratio of 10:1:1. Aqueous solution of $FeSO_4.7\ H_2O$ (0.1 mM to 1.0 mM) was used to prepare a standard calibration curve for the FRAP assay. The assay was started by adding 10 μL of extract to 300 μL of FRAP reagent and the mixture was incubated for 5 min at room temperature. The mixture was then measured at 593 nm against a blank containing only FRAP reagent and 10 μL of water. FRAP values were expressed in mmol of Fe^{2+} equivalents per 100 g of DM of plant sample. Asc and BHT were used as positive controls in this assay.

Table 2: Types of phenolic compounds analyzed by HPLC

Classes of phenolic compounds	Compounds analyzed	Detection wavelength (nm)	Retention time (min)
Hydroxybenzoic acids	Gallic acid	280	5.68
	p-hydroxybenzoic acid		15.13
	Protocatechuic acid		10.07
	Vanilic acid		18.22
Hydroxycinnamic acids	*p*-coumaric acid	320	32.75
	Ferulic acid		37.71
	Chlorogenic acid		19.23
	Caffeic acid		21.21
	Sinapic acid		39.35
	Syringic acid		21.98
Flavonoids	Myricetin	370	48.54
	Rutin		43.84
	Quercetin		48.89

HPLC: High performance liquid chromatography

Anti-lipoxygenase assay

The LOX inhibitory activity was measured based on ferric oxidation of xylenol orange (FOX assay). Anti-LOX activity of the extracts were determined by using a microplate assay modified from.[21] The assay was started by adding 20 μL of extract to 50 μL of 440 ng/mL LOX dissolved in 50 mM Tris-HCl (pH 7.4). The mixture was incubated at room temperature and in the dark for 5 min. Then, 50 μL of 616 μM linoleic acid was added to the mixture, after which the mixture was incubated at room temperature for 20 min in darkness. Next, 100 μL of FOX reagent (15 μM xylenol orange and 15 μM $FeSO_4$ dissolved in a mixture of 15 mL of 300 mM H_2SO_4 and 135 mL of methanol) was added to the mixture. After 30 min of dark incubation, the absorbance of the mixture was measured at 560 nm. Anti-LOX activity was calculated using the formula below:

$$\text{Anti-LOX activity (\%)} = [(A_{control} - A_{Sample})/A_{control})] \times 100$$

$A_{control}$ is the absorbance of the reaction mixture where the plant extract was omitted. A_{sample} is the absorbance of the reaction mixture where the plant extract was added. Extracts

were analyzed in the concentration range of 0-50 mg/mL. EC_{50} value, defined as the extract concentration required to achieve 50% inhibition of LOX activity, was determined by using linear regression analysis. Nordihydroguaiaretic acid was used as the positive control.

Iron chelating assay

This assay was performed in a microplate format, modified from the method described in.[16] First, 80 µL of 0.1 mM $FeSO_4$ was added to 80 µL of plant extract. The mixture was incubated at room temperature for 5 min. Then, 160 µL of 0.25 mM ferrozine was added into each well, followed by 10 min incubation at room temperature. The absorbance of the reaction mixture was measured at 562 nm. Iron chelating activity was calculated using the formula below:

$$\text{Iron chelating (\%)} = [(A_{control} - A_{Sample})/A_{control})] \times 100$$

$A_{control}$ is the absorbance of the reaction mixture where the plant extract was omitted. A_{sample} is the absorbance of the reaction mixture where the plant extract was added. Extracts were analyzed in the concentration range of 0-50 mg/mL. EC_{50} value, defined as the extract concentration required to achieve 50% iron chelating activity, was determined by using linear regression analysis. Disodium ethylenediaminetetraacetic acid (EDTA) was used as the positive control.

Anti-glucosidase assay

The alpha (α)-glucosidase inhibitory activity of the extracts was determined using the procedure described in[22] with slight modifications. The assay was started by mixing 10 µL of extract with 50 µL of 100 mM potassium phosphate buffer (pH 7.0). Subsequently, 30 µL of 0.5 mM 4-nit rophenyl-α-D-glucopyranoside (in 100 mM potassium phosphate buffer, pH 7.0) and 30 µL of 0.1 unit/mL of α-glucosidase (in 10 mM potassium phosphate buffer, pH 7.0) were added to the mixture. The mixture was incubated at 37°C for 30 min. The reaction was terminated by adding 120 µL of 200 mM Na_2CO_3. The absorbance of the reaction mixture was measured at 400 nm. Reaction blanks were prepared by replacing the enzyme with 10 mM phosphate buffer (pH 7.0). Anti-glucosidase activity was calculated using the formula below:

$$\text{Anti-glucosidase activity (\%)} = [(A_{control} - A_{Sample})/A_{control})] \times 100$$

$A_{control}$ is the absorbance of the reaction mixture where the plant extract was omitted. A_{sample} is the absorbance of the reaction mixture where the plant extract was added. Extracts were analyzed in the concentration range of 0-50 mg/mL. EC_{50} value, defined as the extract concentration required to achieve 50% anti-glucosidase activity, was determined by using linear regression analysis. Acarbose, myricetin and quercetin were used as the positive controls.

Data analysis

All experiments were performed in triplicates, and the data are presented as mean ± standard errors. Statistical analysis was performed by using the SAS software version 9.2 (SAS, North Carolina, USA). Data were analyzed using the ANOVA test and means of significant differences ($P < 0.05$) were separated by using Fisher's least significant difference test. Linear regression and correlation analyses were carried out using Microsoft Office Excel 2010 (Microsoft Corporation).

RESULTS

High performance liquid chromatography profiles of phytochemicals

The presence and concentration of four types of HBAs, namely gallic acid (GA), *p*-HBA, vanilic acid (VA) and protocatechuic acid (PCCA), were determined in the extracts of *H. malayana*, *L. adscendens*, and *M. hastata* [Table 3]. Figure 1 shows representative HPLC

Table 3: Hydroxybenzoic acid contents of the macrophyte extracts

Plant	Part	Hydroxybenzoic acids (nmole/g)			
		GA	*p*-HBA	VA	PCCA
H. malayana	Leaf	100.6±3.8$^{a, g}$	712.8±13.0^{a}	166.5±2.8^{a}	331.1±8.5^{a}
	Rhizome	266.7±4.5^{b}	24.2±1.9^{b}	123.0±3.6^{b}	273.3±8.1
L. adscendens	Leaf	46694.4±53.4^{c}	ND	ND	ND
	Stem	8885.7±31.5^{d}	1015.6±14.7^{c}	ND	ND
	Root	3548.3±49.3^{e}	61.7±1.2^{b}	42.4±2.2^{c}	ND
M. hastata	Fruit	687.5±8.4^{f}	1924.5±62.3^{d}	ND	807.2±14.5^{c}
	Leaf	129.0±1.9^{a,b,g}	236.8±2.4^{e}	ND	713.4±4.9^{d}
	Stem	ND	ND	298.0±1.3^{d}	1329.3±33.2^{e}
	Rhizome	174.5±4.7^{b,g}	ND	183.1±4.4^{e}	926.4±14.2^{f}
	Root	ND	ND	ND	ND

Data are presented as mean±SE (n=3). In each column, values followed by different superscript letters are significantly different with P<0.05 as determined by Fisher's LSD test. ND: Undetectable. GA: Gallic acid; *p*-HBA: *p*-hydroxybenzoic acid; VA: Vanilic acid; PCCA: Protocatechuic acid; SE: Standard error; *H. malayana: Hanguana malayana; L. adscendens: Ludwigia adscendens; M. hastata: Monochoria hastata;* LSD: Least significant difference

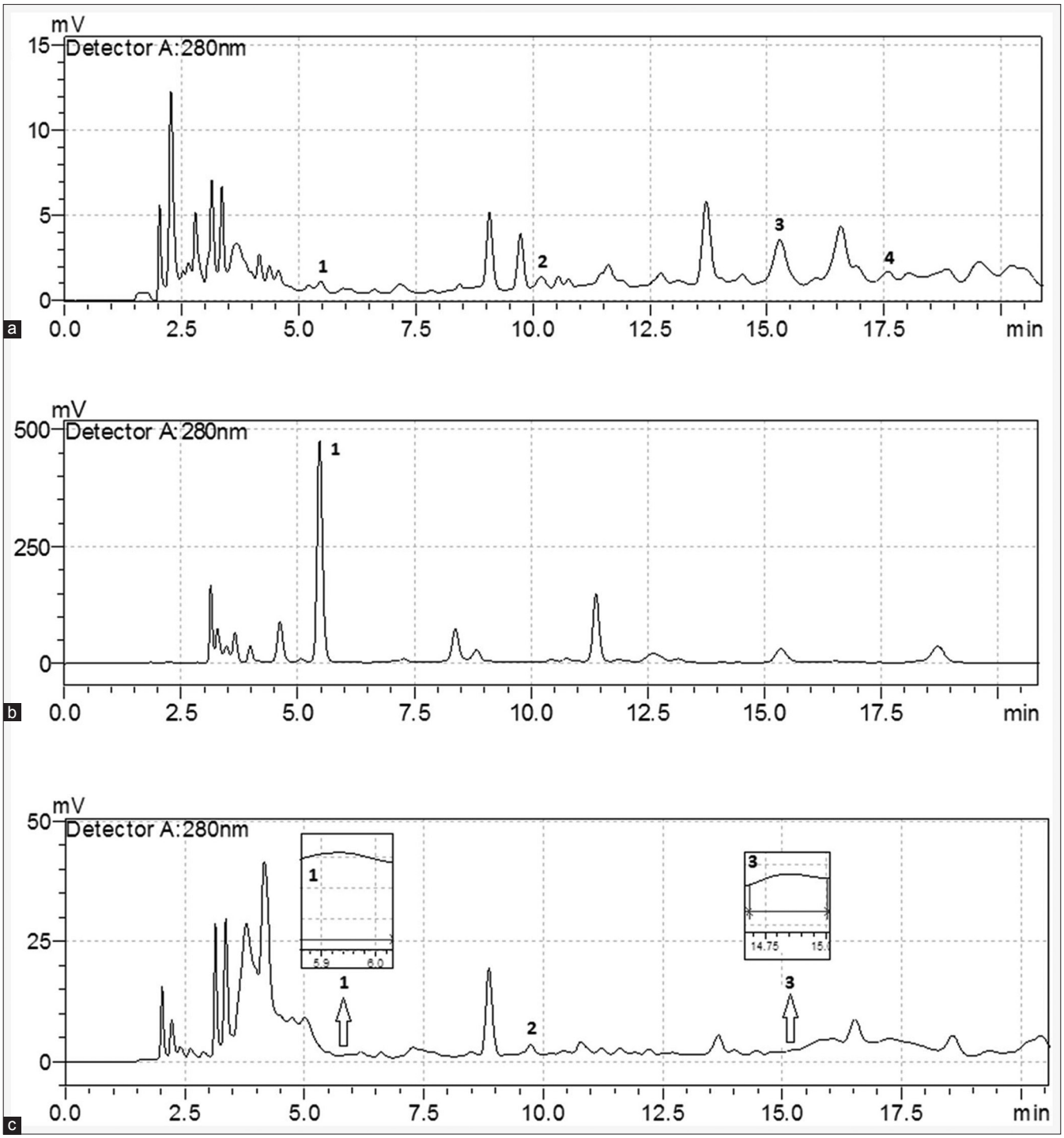

Figure 1: Representative high performance liquid chromatography chromatograms of leaf extracts of (a) *Hanguana malayana* (b) *Ludwigia adscendens* and (c) *Monochoria hastata*. Signals were collected at 280 nm for the detection of hydroxybenzoic acids. (1) Gallic acid; (2) protocatechuic acid; (3) *p*-hydroxybenzoic acid; (4) vanilic acid

chromatograms generated for the detection of the four HBAs in the leaf extracts of the macrophytes. Among the 10 extracts analyzed, only *H. malayana* leaf and rhizome extracts contained all four HBAs. GA was the most abundant HBA, with the highest GA contents detected in the leaf, stem and rhizome extracts of *L. adscendens*. The *L. adscendens* leaf extract contained about 4.7% GA on a plant dry weight basis. The *M. hastata* fruit extract had the highest *p*-HBA concentration, accounting for 0.2% on a dry weight basis. On the other hand, the stem extract of *M. hastata* had the highest VA and PCCA contents.

The concentrations for six hydroxycinnamic acids in the plant extracts, namely, *p*-CA, ferulic acid (FA), chlorogenic

acid (ChA), caffeic acid (CFA), sinapic acid (SNA) and syringic acid (SA), were analyzed [Table 4]. Figure 2 shows representative chromatograms obtained in the HPLC detection of the six hydroxycinnamic acids in the leaf extracts of the macrophytes. Only *H. malayana* leaf and *M. hastata* fruit extracts contained all six hydroxycinnamic acids. On the other hand, *p*-CA was the only hydroxycinnamic acid that was detected in all 10 plant extracts. *H. malayana* leaf extract had the highest FA and SA contents. *L. adscendens* leaf extract had the highest ChA, *p*-CA, and SNA contents. *M. hastata* fruit extract had the highest CFA content.

Among the three flavonoids analyzed, only myricetin was detected in all 10 extracts, ranging between 4.6 and

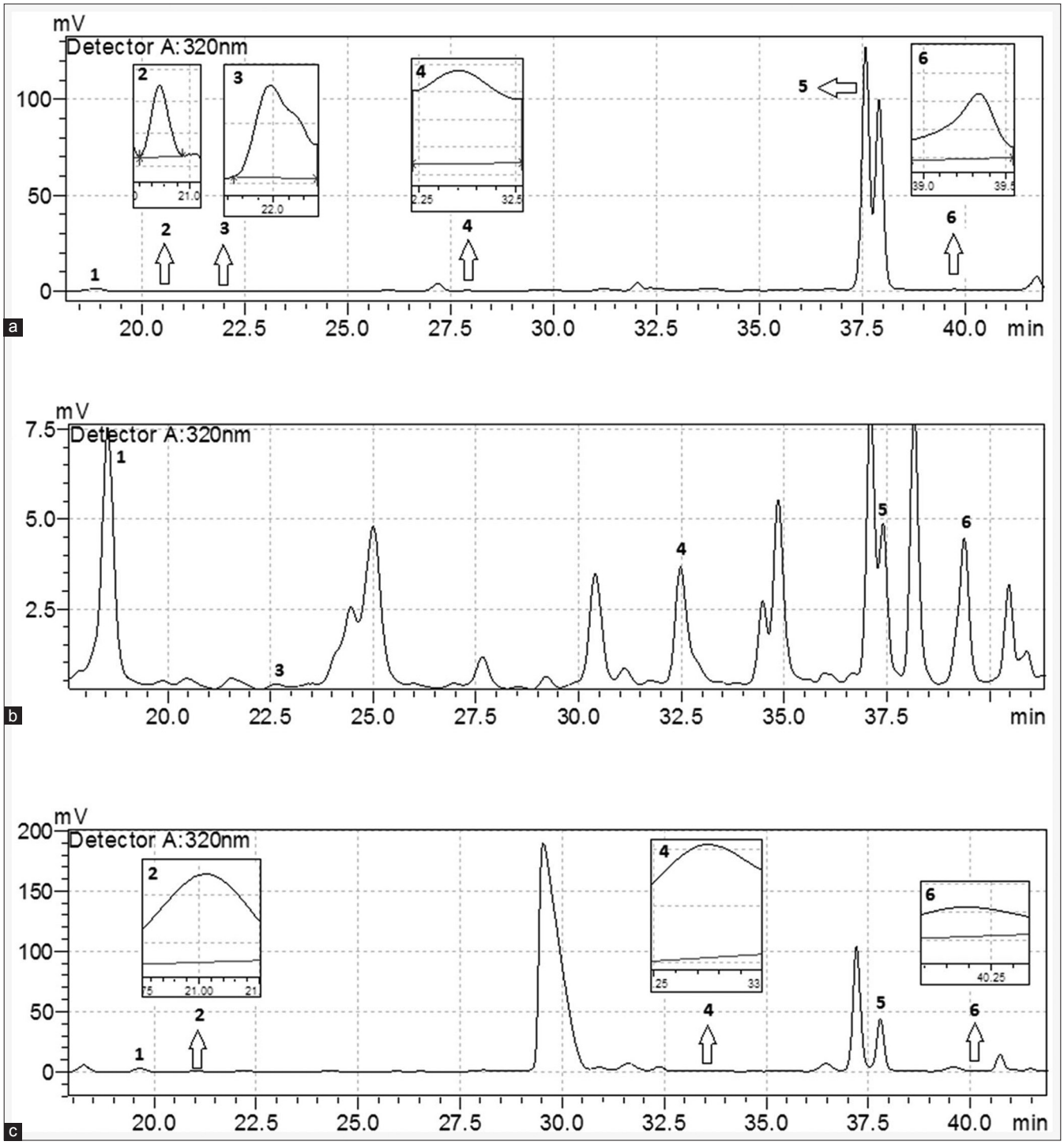

Figure 2: Representative high performance liquid chromatography chromatograms of leaf extracts of (a) *Hanguana malayana,* (b) *Ludwigia adscendens,* and (c) *Monochoria hastata.* Signals were collected at 320 nm for the detection of hydroxycinnamic acids. (1) Chlorogenic acid; (2) caffeic acid; (3) syringic acid; (4) *p*-coumaric acid; (5) ferulic acid; (6) sinapic acid

Table 4: Hydroxycinnamic acid contents of the macrophyte extracts

Plant	Part	Hydroxycinnamic acids (nmole/g)					
		p-CA	FA	ChA	CFA	SNA	SA
H. malayana	Leaf	42.0±0.5^{a}	4272.7±30.7^{a}	122.2±3.1^{a}	11.4±1.1^{a}	29.4±0.2^{a}	2729.4±67.6^{a}
	Rhizome	3.1±0.2^{b}	39.3±0.7^{b,d,e}	34.5±1.0^{b}	ND	ND	ND
L. adscendens	Leaf	254.4±3.3^{c}	343.8±2.7^{c}	830.7±15.5^{c}	ND	410.5±7.5^{b}	1016.5±31.7^{b}
	Stem	21.7±0.2^{d}	47.6±1.6^{d,e}	17.3±0.7^{d}	8.8±0.4^{a}	24.3±0.3^{a}	ND
	Root	10.9±0.2^{e}	ND	9.1±0.3^{d,f}	16.5±0.3^{a,d}	ND	ND
M. hastata	Fruit	15.0±0.5^{f}	62.6±0.8^{d}	32.9±1.1^{b}	141.3±2.6^{b}	15.4±0.2^{c}	1288.7±100.1^{b,c}
	Leaf	33.3±1.8^{g}	1508.3±25.8^{f}	187.3±2.5^{e}	67.3±11.2^{c}	24.2±0.1^{a}	ND
	Stem	66.5±0.7^{h}	302.4±7.7^{g}	11.9±0.2^{d,f}	25.5±0.5^{d}	ND	ND
	Rhizome	2.8±0.1^{b}	21.3±0.4^{b,e,h}	ND	ND	ND	ND
	Root	5.1±0.1^{b}	6.8±0.1^{b,h}	3.5±0.1^{d,f}	17.7±1.1^{a,d}	ND	1115.4±173.4^{c}

Data are presented as means±SE (n=3). In each column, values followed by different superscript letters are significantly different with P<0.05 as determined by Fisher's LSD test. ND: Undetectable; *p*-CA: *p*-coumaric acid; FA: Ferulic acid; ChA: Chlorogenic acid; CFA: Caffeic acid; SNA: Sinapic acid; SA: Syringic acid; SE: Standard error; LSD: Least significant difference; *H. malayana: Hanguana malayana; L. adscendens: Ludwigia adscendens; M. hastata: Monochoria hastata*

2811.2 nmole/g on a plant dry weight basis [Table 5]. Figure 3 shows representative chromatograms obtained in the HPLC analysis of myricetin, rutin and quercetin in the leaf extracts of the macrophytes. In each macrophyte species, higher myricetin content was detected in the leaf extract relative to extracts of other plant parts. Among all 10 extracts, the leaf extract of *L. adscendens* had the highest concentration of myricetin, rutin and quercetin.

Table 5: Flavonoid contents of the macrophyte extracts

Plant	Part	Flavonoid (nmole/g)		
		Myricetin	Rutin	Quercetin
H. malayana	Leaf	575.8±24.5^{a}	126.9±2.5^{a}	5.4±0.2^{a}
	Rhizome	4.6±0.1^{b}	4.0±0.1^{b}	ND
L. adscendens	Leaf	2811.2±17.4^{c}	489.5±3.8^{c}	15.1±0.9^{b}
	Stem	152.1±3.2^{d}	8.1±0.1^{b}	2.4±0.2^{c}
	Root	18.2±0.3^{b}	ND	ND
M. hastata	Fruit	226.4±4.6^{e}	6.5±0.1^{b}	10.8±0.1^{d}
	Leaf	417.5±4.2^{f}	ND	ND
	Stem	173.8±9.2^{d}	5.1±0.4^{b}	3.5±0.2^{e}
	Rhizome	54.3±1.6^{g}	ND	ND
	Root	63.0±1.4^{g}	19.7±0.8^{d}	ND

Data are presented as mean±SE (n=3). In each column, values followed by different superscript letters are significantly different with P<0.05 as determined by Fisher's LSD test. ND: Undetectable; SE: Standard error; *H. malayana: Hanguana malayana; L. adscendens: Ludwigia adscendens; M. hastata: Monochoria hastata*; LSD: Least significant difference

2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

All extracts prepared from *H. malayana*, *L. adscendens*, and *M. hastata* exhibited DPPH radical scavenging activity [Table 6]. The EC_{50} values of the extracts ranged between 0.97 and 66.96 mg/mL. In all three macrophytes, leaf extracts had the lowest EC_{50} values when compared with extracts of other plant parts. The EC_{50} values of the leaf extracts of the three macrophytes, in ascending order, are 0.97 mg/mL (*L. adscendens*), 4.05 mg/mL (*H. malayana*) and 5.08 mg/mL (*M. hastata*). The EC_{50} value of the leaf extract of *L. adscendens* was comparable to those of Asc and BHT; their differences were not statistically different ($P > 0.05$).

Nitric oxide radical scavenging activity

All extracts exhibited NO scavenging activity, with EC_{50} values ranging between 0.31 and 20.80 mg/mL [Table 6]. In the three macrophytes analysed, leaf extracts generally had lower EC_{50} values compared with extracts of other plant parts. In each macrophyte, rhizome and/or root extracts had the highest EC_{50} values. For *M. hastata*, the EC_{50} value of root extract was about 14-fold higher than that of the leaf extract. Notably, statistical analysis revealed that the EC_{50} values of the leaf extracts of *H. malayana* and *L. adscendens* were not significantly different from that of the Asc ($P > 0.05$).

Ferric reducing antioxidant power

All extracts showed ferric reducing ability, with FRAP values ranging between 0.81 and 38.28 mmole Fe^{2+}/100 g DM [Table 6]. The leaf extracts of all three macrophytes showed higher FRAP values compared with extracts of other plant parts. The FRAP values of the leaf extracts also surpassed or resembled the FRAP value of BHT. However, the FRAPvalues of leaf extracts were all lower compared with that of Asc. Among all extracts, the rhizome and root extracts showed the lowest FRAP values.

Anti-lipoxygenase activity

Only stem and root extracts of *L. adscendens*, as well as fruit, leaf and stem extracts of *M. hastata* showed anti-LOX activity [Table 7]. The EC_{50} values of these extracts ranged between 5.90 and 36.96 mg/mL. *M. hastata* leaf extract had the lowest EC_{50} value (5.90 mg/mL) whereas *M. hastata* fruit extract had the highest (36.96 mg/mL). The EC_{50} values of all five anti-LOX extracts were significantly higher than that of nordihydroguaiaretic acid, a LOX inhibitor ($P < 0.05$).

Iron chelating activity

All extracts showed iron chelating activity, with EC_{50} values ranging between 3.24 and 22.93 mg/mL [Table 8]. Leaf and

stem extracts of *L. adscendens* had the lowest EC_{50} values among all the extracts; rhizome extract of *M. hastata* had the highest. In the three macrophytes analyzed, all extracts had significantly higher EC_{50} values compared with disodium EDTA ($P < 0.05$).

Anti-glucosidase activity

Only the extracts of *H. malayana* and *L. adscendens* exhibited α-glucosidase inhibitory activity in the range of extract concentrations tested [Table 9]. Leaf extract of *L. adscendens* had the lowest EC_{50} value (27.5 μg/mL) whereas root extract of the species had the highest (4995.4 μg/mL). Statistical analysis found the EC_{50} value of *L. adscendens* leaf extract to be comparable to those of myricetin and quercetin ($P > 0.05$). The EC_{50} value of the leaf extract was 13-fold lower than that of acarbose, which is an antidiabetic drug with anti-glucosidase activity.

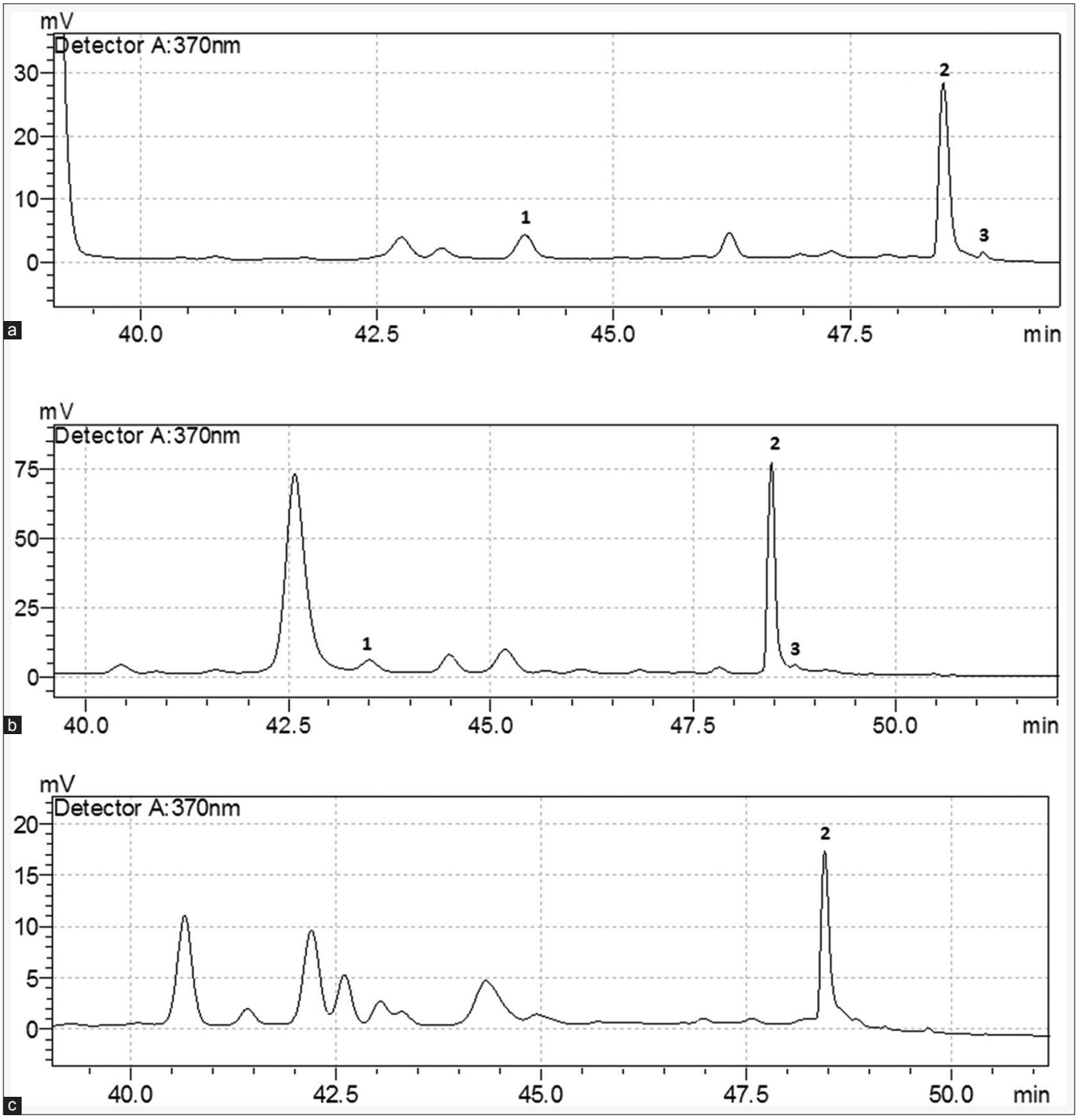

Figure 3: Representative high performance liquid chromatography chromatograms of leaf extracts of (a) *Hanguana malayana,* (b) *Ludwigia adscendens,* and (c) *Monochoria hastata.* Signals were collected at 370 nm for the detection of flavonoids. (1) rutin; (2) myricetin; (3) quercetin

Table 6: EC_{50} values for radical scavenging activities and FRAP values of the macrophyte extracts

Plant	Part	EC_{50} values (mg/mL)		FRAP (mmol Fe^{2+}equivalents/100 g)
		DPPH scavenging activity	NO scavenging activity	
H. malayana	Leaf	4.05±0.02^{a,g}	0.96±0.02^{a,c}	23.60±0.21^{a}
	Rhizome	5.93±0.14^{a}	3.30±0.03^{b}	9.37±0.59^{b,d,e}
L. adscendens	Leaf	0.97±0.00^{b}	0.31±0.00^{c}	38.28±1.64^{c}
	Stem	2.13±0.03^{b,g}	0.73±0.00^{a,c}	27.47±0.09^{c}
	Root	15.42±1.18^{c}	4.84±0.06^{d}	11.19±0.11^{a,b,d,e}
M. hastata	Fruit	33.05±0.74^{d}	11.28±0.13^{e}	4.46±0.14^{d,e}
	Leaf	5.08±0.03^{a}	1.50±0.01^{a}	20.67±0.52^{a,b}
	Stem	32.95±0.15^{d}	13.41±0.31^{f}	10.31±0.30^{a,b,d,e}
	Rhizome	63.82±0.45^{e}	20.64±0.64^{g}	1.59±0.07^{e}
	Root	66.96±2.38^{f}	20.80±0.91^{g}	0.81±0.02^{e}
Positive control	Asc	0.31±0.01^{b}	0.15±0.00^{c}	1029.88±16.72^{f}
	BHT	1.85±0.01^{b,g}	-	17.33±0.49^{a,b,d}

Data are presented as mean±SE (n=3). In each column, values followed by different superscript letters are significantly different with P<0.05 as determined by Fisher's LSD test. Asc: Ascorbic acid; BHT: Butylated hydroxytoluene; *H. malayana: Hanguana malayana; L. adscendens: Ludwigia adscendens; M. hastata: Monochoria hastata*; SE: Standard error; LSD: Least significant difference; EC50: half of maximal effective concentration; FRAP: Ferric reducing antioxidant power; DPPH: 2,2-diphenyl-1-picrylhydrazyl; NO: Nitric oxide

Table 7: EC_{50} values for anti-LOX activity of the macrophyte extracts

Plant	Part	EC_{50} values (mg/mL)
H. malayana	Leaf	ND
	Rhizome	ND
L. adscendens	Leaf	ND
	Stem	24.26±0.09^{a}
	Root	30.82±0.23^{b}
M. hastata	Fruit	36.96±0.34^{c}
	Leaf	5.90±0.02^{d}
	Stem	7.82±0.03^{e}
	Rhizome	ND
	Root	ND
Nordihydroguaiaretic acid (positive control)		0.12±0.00^{f}

Data are presented as mean±SE. Values followed by different superscript letters are significantly different with P<0.05 as determined by Fisher's LSD test. ND: Undetectable; SE: Standard error, *H. malayana: Hanguana malayana; L. adscendens: Ludwigia adscendens; M. hastata: Monochoria hastata*; SE: Standard error; LSD: Least significant difference; EC_{50}: Half of maximal effective concentration; LOX: Lipoxygenase

Table 8: EC_{50} values for iron chelating activity of the macrophyte extracts

Plant	Part	EC_{50} values (mg/mL)
H. malayana	Leaf	5.30±0.07^{a}
	Rhizome	5.24±0.04^{a}
L. adscendens	Leaf	3.24±0.04^{b}
	Stem	3.37±0.04^{b}
	Root	20.15±0.49^{c}
M. hastata	Fruit	7.28±0.09^{d}
	Leaf	6.57±0.00^{d}
	Stem	12.02±0.26^{e}
	Rhizome	22.93±0.59^{f}
	Root	21.21±0.71^{g}
Disodium EDTA (positive control)		0.02±0.00^{h}

Data are presented as mean±SE. Values followed by different superscript letters are significantly different with P<0.05 as determined by Fisher's LSD test. *H. malayana: Hanguana malayana; L. adscendens: Ludwigia adscendens; M. hastata: Monochoria hastata*; SE: Standard error; LSD: Least significant difference; EDTA: Ethylenediaminetetraacetic acid; EC_{50}: Half of maximal effective concentration

Correlation analysis

p-coumaric acid, GA, and myricetin were detected in all or most extracts. Hence, we analyzed their correlations with bioactivities of the extracts. Overall, *p*-CA, GA, and myricetin contents were strongly correlated with DPPH and NO scavenging activities as well as anti-glucosidase activity of the plant extract [Table 10]. Notably, when compared with *p*-CA and myricetin, GA content was correlated more strongly with these bioactivities (R^2 = 0.84-0.97). There were weak or no statistically significant correlations between these phytochemical parameters and other bioactivities investigated. On the other hand, we also found iron chelating activity to be correlated with DPPH scavenging activity (R^2 = 0.69) and with NO scavenging activity (R^2 = 0.65).

DISCUSSION

Phytochemical profiling by high performance liquid chromatography

Our study found wetland macrophytes, *H. malayana*, *L. adscendens*, and *M. hastata*, to differ in their phytochemical profiles in both qualitative and quantitative manners. For example, when leaf extracts of the three species were compared, only *H. malayana* contained all four HBAs and six hydroxycinnamic acids analyzed. For HBAs, *p*-HBA, VA and PCCA were undetectable in the leaf extract of *L. adscendens*; VA was not found in the leaf extract of *M. hastata*. For hydroxycinnamic acids, CFA and SA were not found in the leaf extracts of *L. adscendens* and *M. hastata*, respectively. On the other hand, although all three types of flavonoids analyzed were present in the leaf extracts of

Table 9: EC_{50} values for anti-glucosidase activity of *H. malayana* and *L. adscendens* extracts

Plant	Part	EC_{50} values (μg/mL)
H. malayana	Leaf	797.3±5.6[a]
	Rhizome	850.5±1.7[b]
L. adscendens	Leaf	27.5±0.1[c]
	Stem	88.7±0.4[d]
	Root	4995.4±30.8[e]
Positive control	Acarbose	359.1±0.8[f]
	Myricetin	34.7±0.3[c]
	Quercetin	37.0±0.0[c]

Data are presented as mean±SE. Values followed by different superscript letters are significantly different with $P<0.05$ as determined by Fisher's LSD test. None of the extracts prepared from *M. hastata* showed any anti-glucosidase activity; hence EC_{50} values were not calculated for the species. *H. malayana: Hanguana malayana; L. adscendens: Ludwigia adscendens*; SE: Standard error; EC_{50}: Half of maximal effective concentration; LSD: Least significant difference; *M. hastata: Monochoria hastata*

Table 10: Correlation analysis between selected phytochemical contents and bioactive parameters

Bioactive parameters ($1/EC_{50}$ values)	Coefficient of determination (R^2) Phytochemical contents		
	p-CA	GA	Myricetin
DPPH scavenging activity	0.74	0.87	0.79
NO scavenging activity	0.75	0.84	0.83
FRAP*	0.48	0.49	0.51
Anti-LOX activity	NS	NS	NS
Iron chelating activity	NS	0.35	NS
Anti-glucosidase activity	0.82	0.97	0.84

Values presented are all statistically significant ($P<0.05$). *p*-CA: *p*-coumaric acid; GA: Gallic acid; NS: Not statistically significant; FRAP: Ferric reducing antioxidant power; LOX: Lipoxygenase; DPPH: 2,2-diphenyl-1-picrylhydrazyl; EC_{50}: Half of maximal effective concentration; NO: Nitric oxide. *FRAP values were used instead of $1/EC_{50}$ values

the macrophytes, their quantitative profiles differed. For example, the leaf extract of *L. adscendens* contained 4.9-fold and 6.7-fold greater myricetin content than the leaf extracts of *H. malayana* and *M. hastata,* respectively.

Examination of the three macrophytes found leaves to be the most prominent source of phytochemicals from the classes of HBAs, hydroxycinnamic acids and flavonoids. This observation agrees with previous investigations which compared the phytochemical profiles of extracts prepared from different organs of medicinal plants.[23-25] Notably, *L. adscendens* leaf extract was found to be the richest source of *p*-CA, GA, and myricetin among the ten extracts prepared from the three macrophytes. The presence of these three phytochemicals in *L. adscendens* has not been previously reported in the literature. To the best of our knowledge, this is also the first report of the HPLC profiles of HBAs, hydroxycinnamic acids and flavonoids in *H. malayana* and *M. hastata.*

P-coumaric acid has been shown to protect against oxidation of low-density lipoprotein cholesterol,[26] to improve the conditions of type II diabetes and insulin resistance by modulating glucose and lipid metabolism,[8] as well as reducing carcinogenic nitrosamines formation, which would be beneficial in preventing colon cancer.[27] GA is known to induce apoptosis in various cancer cell lines.[28,29] It is considered beneficial to cancer treatment because it is selectively toxic to cancerous cells and relatively nontoxic to normal cells.[30] Myricetin, on the other hand, has chemopreventive effect on skin cancer[31] and exhibits anti-inflammatory and antidiabetic activities.[32] The presence of such health-promoting and therapeutically-relevant phytochemicals highlights the value of *L. adscendens* as a source of potential therapeutic agents.

Based on the profile of 13 selected phenolic phytochemicals analyzed, the most abundant types of HBAs, hydroxycinnamic acids and flavonoids in both *H. malayana* and *M. hastata* were *p*-HBA, FA, and myricetin, respectively. *H. malayana* leaf extract had the highest FA and SA contents among all ten extracts. Meanwhile, *M. hastata* fruit extracts had the highest *p*-HBA content among all extracts respectively. *p*-HBA, FA, and myricetin are all known to have therapeutically-relevant effects such as prevention of lipid peroxidation,[33] reduction of inflammatory markers nuclear factor-kappa β and COX-2[9] and antidiabetic effects.[32] Our results thus highlight that in addition to the relatively well-studied *L. adscendens*, *H. malayana* and *M. hastata* also deserve more attention as a source of health-promoting natural products.

Biological activities

Our study demonstrated that *H. malayana*, *L. adscendens*, and *M. hastata* are potential resources of bioactive phytoconstituents. Extracts of all three plants showed antioxidant and iron chelating activities. Anti-glucosidase activity was detected only in *H. malayana* and *L. adscendens*. We also detected anti-LOX activity in some extracts of *L. adscendens* and *M. hastata*. Notably, *L. adscendens* had potent antioxidant and anti-glucosidase activities which were comparable to those of the reference compounds. Importantly, this is the first report of anti-glucosidase activity in *L. adscendens*. This is also the first time anti-LOX activity is reported for *L. adscendens* and *M. hastata*.

Antioxidant parameters (DPPH and NO scavenging activities) were found to be positively and significantly correlated with selected phytochemical contents (*p*-CA, GA, and myricetin). This suggests that the antioxidant activities of the extracts analyzed can be attributed at least in part to the presence of *p*-CA, GA, and myricetin. Our finding of such a strong correlation in the macrophyte extracts is also supported by reports of antioxidant activity of the three phenolic compounds.[7,34,35] Such a correlation also provides a plausible explanation for *L. adscendens* leaf

extract having the highest levels of antioxidant activities among all 10 extracts.

In this study, leaf extracts showed higher antioxidant activity compared to extracts of other plant parts. This finding corresponds well with our observation of the overall higher abundance of phenolic constituents in leaf extracts relative to other extracts. Prominent antioxidant activity in leaf extracts relative to extracts of other parts of the same plant has been previously reported.[23-25] Close and McArthur[36] proposed that the abundance of antioxidant phenolic constituents in leaf tissues may be attributed to their biological needs to protect themselves against photosynthesis-associated photooxidative stress.

Similar to radical scavenging activity, iron chelating activity was detected in all ten extracts prepared from the three macrophytes. Iron chelating agents may act as secondary antioxidants owing to their ability to chelate iron, which could catalyze and accelerate the Haber-Weiss and Fenton reaction, leading to the production of hydroxyl radicals.[37] We also observed a correlation between iron chelating and radical scavenging activities among the extracts. Our results suggest that the plant extracts may contain antioxidant compounds with concurrent radical scavenging and iron chelating activities. This possibility is plausible as our phytochemical analysis revealed the presence of phenolic constituents with concurrent radical scavenging and iron chelating activities in the macrophytes. Myricetin, for example, exhibits strong radical scavenging and metal chelating activities.[11,38] The potential application and benefits of antioxidants with iron chelating properties in the management of iron-related human diseases have been highlighted in a recent review.[37] Leaf extract of *L. adscendens*, which possessed the highest radical scavenging activity, also exhibited the highest iron chelating activity. Hence, *L. adscendens* is the most promising candidate from which to isolate such antioxidants.

Anti-LOX activity was only detectable in selected extracts of *L. adscendens* and *M. hastata* in this study. There is no clear correlation between the anti-LOX activity of the extracts and their phytochemical contents. A similar lack of correlation between anti-LOX activity and phenolic contents in red and white wine extracts was previously reported.[39] Our results imply that anti-LOX activity and LOX-inhibitory phytoconstituents are relatively less ubiquitous compared with antioxidant and iron chelating compounds. Based on EC_{50} values, the leaf and stem extracts of *M. hastata* are the most promising anti-LOX agents among all extracts analyzed. Boils is caused by localized skin bacterial infection which starts with itching and is followed by inflammation.[40] 5-LOX is one of the inflammatory mediators.[41] Hence, our finding of anti-LOX activity in *M. hastata* leaf extract substantiates the traditional uses of the plant in the treatment of boils. Further work to isolate and purify anti-LOX constituents from the species is desirable.

The EC_{50} value for the anti-glucosidase activity of *L. adscendens* leaf extract is lower than that of acarbose and comparable to those of myricetin and quercetin. This indicates that the extract possessed very strong anti-glucosidase activity. *L. adscendens* stem extract also exhibited fairly strong anti-glucosidase activity. The stem extract had an EC_{50} value for anti-glucosidase activity that is, although higher than those of myricetin and quercetin, still lower compared with acarbose. *L. adscendens* is not traditionally used for treating diabetes, but in some parts of India and China, the macrophyte is consumed as a vegetable.[42,43] An animal study revealed that ethyl acetate extract of *L. adscendens* had hypoglycemic effects in alloxan-induced diabetic rats.[43] This finding, together with our observation of the potent anti-glucosidase activity in the aqueous extracts of *L. adscendens,* suggests that the plant may have potent antidiabetic or antihyperglycemic properties when consumed.

Based on our results on *L. adscendens* and *H. malayana*, leaves are a more prominent source of anti-glucosidase agents compared with other plant parts. Our observation is in line with the estimation that 35% of antidiabetic phytoconstituents are stored in the leaf, while the rest are distributed at lower percentages across different plant parts.[44] Among the 10 extracts analyzed, leaf extract of *L. adscendens*, which had the strongest anti-glucosidase activity, also had the highest contents of *p*-CA, GA, and myricetin. We also found anti-glucosidase activity of the extracts to be positively correlated with *p*-CA, GA, and myricetin contents. Hence, the three compounds likely contribute to at least some of the anti-glucosidase effects seen in the extracts of *L. adscendens* and *H. malayana*. Further supporting this proposal are previous reports of the glucosidase inhibitory activity of *p*-CA,[45] GA[46,47] and myricetin.[48]

CONCLUSIONS

Our study has demonstrated the multiple bioactivities of wetland macrophytes, *H. malayana*, *L. adscendens*, and *M. hastata*. We found antioxidant and iron chelating activities in all extracts analyzed. Anti-glucosidase activity was detected only in *H. malayana* and *L. adscendens*, whereas anti-LOX activity was found in some extracts of *L. adscendens* and *M. hastata*. HPLC analysis found that the macrophytes differed in their phytochemical profiles,

but *p*-CA, GA, and myricetin were detected in all or most of the extracts. Leaves of the macrophytes were the most prominent source of health-promoting phytochemicals and bioactivities. Notably, *L. adscendens* leaf extract, which had the highest *p*-CA, GA, and myricetin contents, also exhibited strong antioxidant and anti-glucosidase activities that were comparable to the reference compounds.

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